The Divergent Orphan Nuclear Receptor ODR-7 Regulates Olfactory Neuron Gene Expression via Multiple Mechanisms in *Caenorhabditis elegans*

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ABSTRACT

Nuclear receptors regulate numerous critical biological processes. The *C. elegans* genome is predicted to encode ~270 nuclear receptors of which >250 are unique to nematodes. ODR-7 is the only member of this large divergent family whose functions have been defined genetically. ODR-7 is expressed in the AWA olfactory neurons and specifies AWA sensory identity by promoting the expression of AWA-specific signaling genes and repressing the expression of an AWC-specific olfactory receptor gene. To elucidate the molecular mechanisms of action of a divergent nuclear receptor, we have identified residues and domains required for different aspects of ODR-7 function *in vivo*. ODR-7 utilizes an unexpected diversity of mechanisms to regulate the expression of different sets of target genes. Moreover, these mechanisms are distinct in normal and heterologous cellular contexts. The *odr-7* ortholog in the closely related nematode *C. briggsae* can fully substitute for all ODR-7-mediated functions, indicating conservation of function across 25–120 million years of divergence.

NUCLEAR receptors (NRs) are an important family of transcriptional regulators that have been implicated in diverse biological processes including embryonic and neuronal development, insect metamorphosis, sexual differentiation, metabolic regulation, and in the response to xenobiotics (Thummel 1995; Chawla *et al.* 2001; Garcion *et al.* 2002; Willson and Kliewer 2002). The NR superfamily includes proteins whose transcriptional functions are modulated by binding small molecules such as steroids, retinoids, and fatty and bile acids (Mangelsdorf *et al.* 1995; Chawla *et al.* 2001). However, many members of this superfamily are so-called "orphan" receptors for which ligands either do not exist or have not yet been identified (Giguere 1999; Kliewer *et al.* 1999). Interestingly, NRs have been identified only in metazoans, indicating that these molecules are likely to play critical roles in a multicellular context.

Members of the NR family generally contain four well-defined domains, including the DNA-binding domain (DBD) and ligand-binding domain (LBD; see Freedman 1997 for comprehensive reviews). The highly conserved DBD consists of two zinc fingers and represents the signature domain of this superfamily. Primarily on the basis of sequence homology in the DBD and LBD, NRs have been placed into six classes (Laudet 1997). Five of these classes include NRs from multiple phyla, indicating that these NRs arose early in evolution (Laudet 1997; Sluder and Maina 2001). However, to date, the steroid receptor family has been identified only in vertebrates.

The complete genome sequence of *Caenorhabditis elegans* revealed a plethora of predicted NRs. The human and the Drosophila genomes are predicted to encode ~48 and ~21 NRs, respectively, whereas the *C. elegans* genome is predicted to encode ~270 members of the NR family, representing the largest family of transcriptional regulators in the worm genome (Sluder *et al.* 1999; Maglich *et al.* 2001; Sluder and Maina 2001). Approximately 15 of the predicted *C. elegans* NRs represent members of classes that are conserved across phyla, and several of these genes have been shown to play critical roles in multiple aspects of development (Sluder and Maina 2001). The remaining >250 NRs are divergent and appear not to be represented in non-nematode species (Sluder *et al.* 1999). Although several members of this large divergent NR family have been shown to be expressed in multiple tissue types in *C. elegans* (Miyabayashi *et al.* 1999; Sluder *et al.* 1999), to date the functions of only the *odr-7*-encoded divergent NR have been described genetically (Sengupta *et al.* 1994). Thus, the roles of these unusual NRs, as well as their mechanisms of action, remain to be elucidated.

Knowledge of ODR-7 functions provides us with a unique opportunity to dissect the molecular mechanisms of a divergent NR function *in vivo*. ODR-7 contains a DBD characteristic of the NR family located at the C terminus of the protein (Sengupta *et al.* 1994). However, additional domains are not conserved. *odr-7* is expressed exclusively in the bilateral AWA olfactory neurons that are required for the attractive responses of *C. elegans* to a panel of volatile odorants including

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diacetyl and pyrazine (Bargmann *et al.* 1993; Sengupta *et al.* 1994). *odr-7* expression is initiated by the LIM homeobox gene *lin-11* and is maintained by autoregulation (Sarafi-Reinach *et al.* 2001). Animals carrying null mutations in *odr-7* fail to express AWA-specific signaling genes (Sengupta *et al.* 1996) and fail to respond to all odorants sensed by the AWA neurons (Sengupta *et al.* 1994). In *odr-7* null mutants, the AWA neurons instead ectopically express the *str-2* olfactory receptor, which is normally expressed asymmetrically in either the left or the right AWC olfactory neuron (Sagasti *et al.* 1999; Troemel *et al.* 1999). Thus, ODR-7 plays a critical role in determining the sensory specificity of the AWA neurons by activating the expression of AWA-specific genes and by repressing the expression of AWC-specific signaling genes.

Here, we perform an *in vivo* analysis of the residues and domains required for ODR-7-mediated activation and repression of target genes and demonstrate that ODR-7 utilizes multiple mechanisms for the regulation of gene expression. *C. elegans* and the closely related nematode *C. briggsae* are thought to have diverged 25–120 million years ago. We find that all residues and domains identified as essential for ODR-7 functions are conserved in the *C. briggsae* ODR-7 ortholog, which can fully substitute for all ODR-7-mediated functions in *C. elegans*.

MATERIALS AND METHODS

Strains and genetics: Worm strains were grown under standard conditions (Brenner 1974). Strains carrying stably integrated *str-2*p*::gfp* and *odr-7*p*::gfp* fusion genes were the following: *kyIs140 (str-2*p*::gfp)* I (PY1113) (Troemel *et al.* 1999) and *kyIs38 (odr-7*p*::gfp)* X (PY1060) (Sengupta *et al.* 1994).

Molecular biology: Standard molecular biology techniques were used (Sambrook *et al.* 1989). *C. briggsae odr-7* genomic sequences were isolated from genomic DNA by amplification. The *odr-7* minigene was generated by driving a full-length *odr-7* cDNA lacking the SL1 splice acceptor site and including 42 bp of 3′ untranslated region sequences under the *odr-7* promoter (Sengupta *et al.* 1994). To bypass the requirement for autoregulation in the AWA neurons, we also expressed the *odr-7* cDNA under the *osm-6* promoter, which drives expression in most ciliated neurons in *C. elegans*, including the AWA neurons (Collet *et al.* 1998). However, this fusion gene failed to rescue the gene expression defects of *odr-7(ky4)* animals.

Site-directed mutagenesis was carried out with the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Following mutagenesis, *odr-7* cDNAs were sequenced prior to being cloned into the appropriate expression vectors. Domain deletions were generated by digestion with the appropriate restriction enzymes and religation. Junctions were confirmed by sequencing. A cDNA encoding NHR-74 was kindly provided by Marc van Gilst. Further details of plasmid constructions and primer sequences used are available upon request.

Behavioral assays: Population chemotaxis assays were performed as described previously (Bargmann *et al.* 1993). Statistical significance was determined using the Bonferroni-Dunn multiple-comparisons procedure (StatView; Abacus Concepts, Berkeley, CA).

Germ-line transformations: Germ-line transformations were carried out using standard protocols (Mello and Fire 1995). Coinjection markers used were either the dominant pRF4 *rol-6 (su1006)* marker at 100 ng/μl or the *unc-122*p*::gfp* marker at 30 ng/μl (Miyabayashi *et al.* 1999). *unc-122*p*::gfp* was used as the coinjection marker to generate all strains whose chemosensory behaviors were analyzed. All plasmids were injected at 15 ng/μl, except for the *odr-1*p*::odr-7* construct, which was injected at 50 ng/μl to generate the *kyIs140*::Ex*[odr-1*p*::odr-7]* strains whose chemosensory responses are shown in Figure 4B.

Immunofluorescence and microscopy: Staining with anti-ODR-7 antibodies was carried out as described previously (Sarafi-Reinach *et al.* 2001). Where applicable, animals were examined by epifluorescence using a Zeiss Axioplan microscope, and images were captured using a CCD camera (Hamamatsu, Bridgewater, NJ). Images were analyzed using Openlab (Improvision) and Adobe Photoshop (Adobe Systems) software.

RESULTS

An *odr-7* "minigene" rescues *odr-7* mutant phenotypes: We generated an *odr-7* minigene by driving the *odr-7* cDNA under the *odr-7* promoter (see MATERIALS AND METHODS). This minigene fully restored the ability of *odr-7(ky4)* mutants to respond to both diacetyl and pyrazine (Figure 3). Expression of this minigene in *odr-7(ky4)* animals also repressed the ectopic expression of a *str-2*p*::gfp* reporter gene (henceforth referred to as *str-2*) in the AWA neurons (Figure 2). *odr-7(ky55)* mutants carry a missense mutation in a highly conserved residue in the DBD of ODR-7 (G340E; Figure 1A) and fail to respond specifically to diacetyl, while retaining wild-type responses toward pyrazine (Sengupta *et al.* 1994). *odr-7 (ky55)* mutants also fail to ectopically express *str-2* in the AWA neurons (Sagasti *et al.* 1999). The *ky55* mutation is unlikely to result in reduced levels of ODR-7 protein since staining *odr-7(ky55)* animals with anti-ODR-7 antibodies showed wild-type levels of expression (P. Sengupta, unpublished observations). Moreover, *odr-7(ky55)/ odr-7(ky4) trans*-heterozygous animals also retained the ability to respond to pyrazine while failing to respond to diacetyl (data not shown), indicating that the *ky55* mutation specifically affects a subset of ODR-7-mediated functions. Using site-directed mutagenesis, we created a point mutation in the *odr-7* minigene that is predicted to result in a G340E substitution. *odr-7* null mutant animals carrying this mutated minigene phenocopied *odr-7 (ky55)* animals in that they were rescued for their behavioral responses toward pyrazine but not diacetyl (Figure 3) and were also rescued for the *str-2* misexpression phenotype (Figure 2). Taken together, these results indicate that expression of the *odr-7* minigene accurately reflects the functions of the endogenous *odr-7* locus and that this minigene may be utilized to identify residues and domains essential for ODR-7-mediated functions.

Maintenance of *odr-7* expression via autoregulation requires residues in the N-terminal domain of ODR-7 and in the DBD: We first determined the requirements for ODR-7 to maintain its own expression. We created

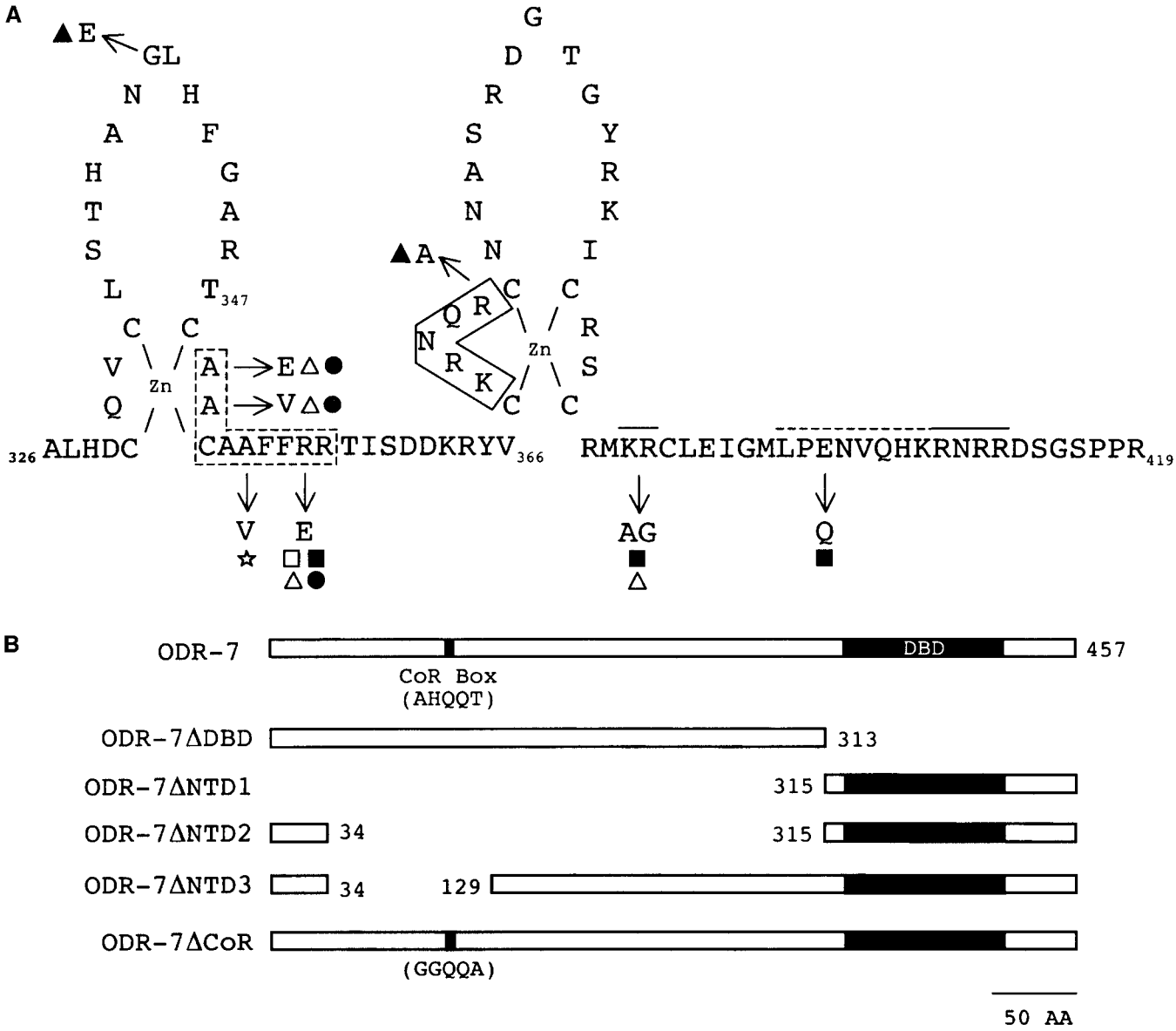


FIGURE 1.—Residues and domains mutated in ODR-7. (A) The predicted DBD of ODR-7 is shown. Residues boxed with dashed lines comprise the P box and the basic quartet, residues boxed with solid lines comprise the D box, basic residues comprising the putative NLS are shown with a solid overbar, residues comprising the predicted but poorly conserved T box are shown with a dashed overbar. The missense mutations analyzed in this work are indicated. The following symbols denote the functions affected by missense mutations at the indicated residues: ●, autoregulation; △, chemotaxis to diacetyl and pyrazine; ▲, chemotaxis to diacetyl; □, repression of *str-2* expression in the AWA neurons; ■, repression of *str-2* expression in the AWC neurons; ☆, no effect. See text for additional details. (B) Domains deleted in each construct are shown. The AHQQT motif in the putative CoR box was mutated to GGQQA in ODR-7ΔCoR.

point mutations and deletions in the *odr-7* cDNA and investigated whether the mutant proteins were able to maintain expression of *odr-7* in *odr-7(ky4)* null mutants by staining with anti-ODR-7 antibodies or by their ability to maintain expression of an *odr-7*p*::gfp* transgene. Deletion of either the DBD (ODR-7Δ DBD) or the N-terminal domain (NTD; ODR-7ΔNTD1) resulted in a failure to maintain *odr-7*p*::gfp* expression (Figure 2), suggesting that sequences in both the DBD and the N terminus of ODR-7 may be required for autoregulation. Although we are unable to exclude the possibility that the failure to autoregulate results from loss of stability or mislocalization of these mutant proteins in the AWA neurons, both ODR-7Δ DBD and ODR-7ΔNTD1 are able to repress *str-2* expression in the AWA neurons (Figure 2; see below), suggesting that these mutant proteins retain a subset of ODR-7 functions.

To further delineate the residues in the NTD required for autoregulation, we examined the effects of expressing two additional N-terminal deletions, ODR-7ΔNTD2 and ODR-7ΔNTD3 (Figure 1B). Neither deletion mutant was able to autoregulate (Figure 2). Thus, in addi-

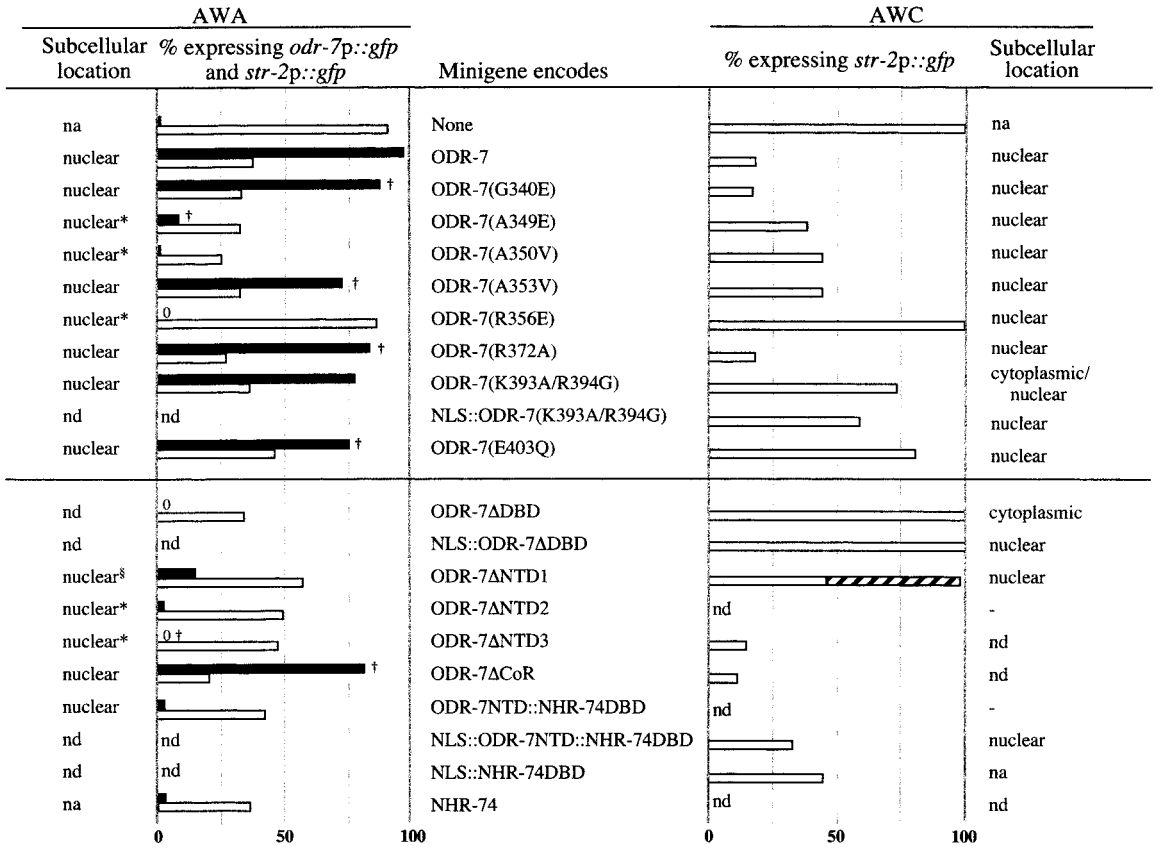


FIGURE 2.—Regulation of *odr-7* and *str-2* expression by ODR-7 mutants. *odr-7* plasmids injected into *odr-7* null mutants (left) or wild-type (right) animals are indicated at center. The cDNAs were expressed under the *odr-7* (left) or *odr-1* (right) promoters. The subcellular localization of encoded mutant proteins in the AWA and AWC neurons as detected by staining with anti-ODR-7 antibodies is indicated. *, expression observed in larvae but not in adults; §, expression detected by tagging with GFP. (Left) For each strain, shown is the percentage of transgenic animals able to maintain *odr-7* expression in at least one AWA neuron (solid bars) as detected by staining with anti-ODR-7 antibodies (indicated by †) or expression of an integrated *odr-7*p::*gfp* transgene. $n > 40$ for each; data from two independent transgenic lines are shown. The percentage of transgenic animals misexpressing an integrated *str-2*p::*gfp* transgene in at least one AWA neuron is shown (open bars). $n > 95$ for each; data from two transgenic lines are shown. ODR-7 expression levels in each transgenic line were comparable to those of lines expressing wild-type ODR-7. (Right) The percentage of transgenic animals expressing an integrated *str-2*p::*gfp* transgene in a single AWC neuron is shown. $n > 95$ for each; data from two transgenic lines are shown. For ODR-7ΔNTD1, the hatched bar represents the percentage of transgenic animals expressing *str-2* in both AWC neurons. For each plasmid, only lines in which mutant ODR-7 was expressed in the AWC neurons at levels comparable to those of lines expressing wild-type ODR-7 in the AWC neurons were quantitated. na, not applicable; nd, not done. Independent transgenic lines generated with each plasmid exhibited equivalent phenotypes.

tion to residues in the DBD, residues included in the NTD between amino acids (aa) 35–128 may also be required for autoregulation. Unliganded NRs bind to corepressor proteins such as N-CoR via an AHXXT motif in the "CoR" box in their LBDs (CHEN and EVANS 1995; HORLEIN *et al.* 1995; KUROKAWA *et al.* 1995). We identified a similar AHQQT motif in the domain deleted in ODR-7ΔNTD3 (Figure 1B). Although the *C. elegans* genome is not predicted to encode a homolog of N-CoR, we nevertheless mutated the AHQQT motif in the full-length ODR-7 protein (ODR-7ΔCoR). ODR-7ΔCoR fully rescued all *odr-7* null phenotypes (Figures 2 and 3), indicating that residues in this domain in addition to or other than this motif are essential for the ability of ODR-7 to autoregulate.

We next identified residues in the DBD required for autoregulation. P-box residues in the first zinc finger are required for the recognition and discrimination of specific sequences in the cognate DNA-binding site (DANIELSEN *et al.* 1989; MADER *et al.* 1989; UMESONO and EVANS 1989; LUISI *et al.* 1991), whereas a quartet of highly conserved basic residues, FF(K/R)R, has been shown to contact both specific bases of the binding site as well as the phosphate backbone of DNA (HARD *et al.* 1990; LUISI *et al.* 1991; RASTINEJAD *et al.* 1995). We identified three missense mutations that abolished the ability of ODR-7 to maintain its own expression by autoregulation (Figure 2). These included the A349E and A350V mutations in the P box and the R356E mutation in the conserved FFRR basic quartet (Figure 1A). The *odr-7(oy43)* allele encodes a protein with an A350V mutation and exhibits a similar defect in autoregulation (T.

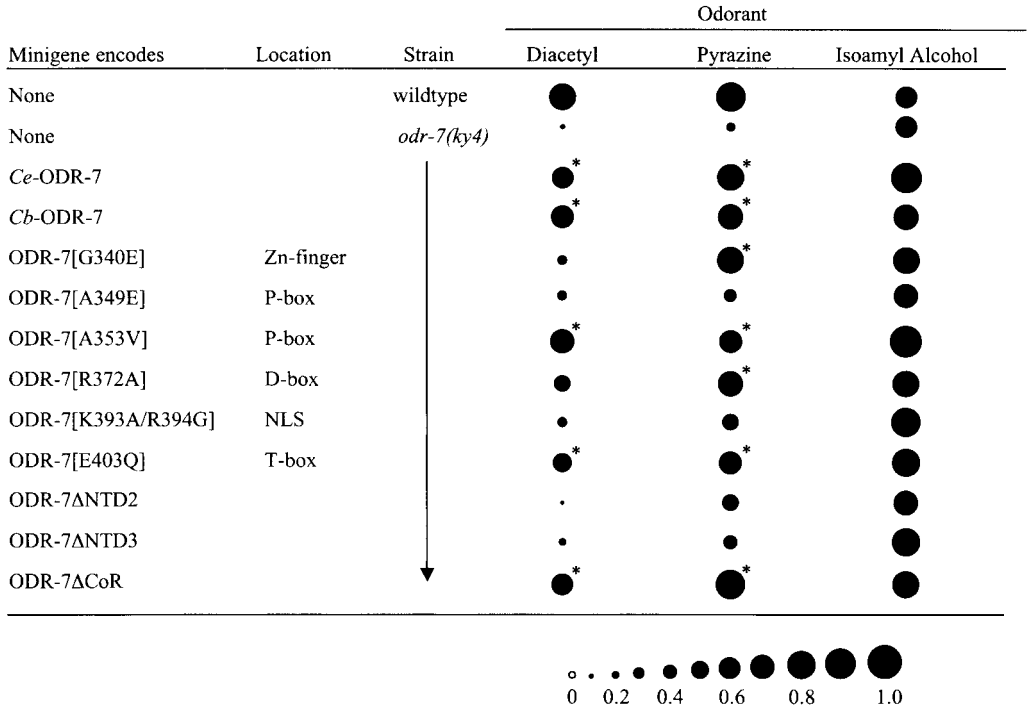


FIGURE 3.—Chemosensory responses of ODR-7 mutants. Diacetyl and pyrazine are AWA-sensed odorants; isoamyl alcohol is sensed by the AWC neurons. The sizes of the circles represent the chemotaxis index as indicated at bottom. Chemotaxis indices range from 0 to 1.0, where 0 represents no attraction and 1.0 represents complete attraction (BARGMANN *et al.* 1993). Concentrations of odorants used at the peak of the gradients were 1 nl of diacetyl, 1 μl of 10 mg/ml pyrazine, and 1 μl of 1:100 dilution of isoamyl alcohol. All plasmids were injected into a *odr-7(ky4)* strain carrying integrated copies of a *str-2*p*::gfp* transgene using *unc-122*p*::gfp* as the coinjection marker. The locations of the missense mutations are indicated. Each data point represents the mean of the responses of ~80–100 transgenic animals from at least two independent lines assayed on 2 days. The responses of each line were equivalent. Standard error values were 0.01–0.19 of the mean. Responses significantly different from those of *odr-7(ky4)* animals at $P < 0.05$ are indicated by an asterisk.

MELKMAN and P. SENGUPTA, unpublished results). In all cases, nuclear expression of the mutant ODR-7 proteins was detected in the AWA neurons of early larvae (Figure 2 and data not shown), and multiple transgenic lines exhibited similar phenotypes, suggesting that the autoregulatory defects may not arise simply as a consequence of lack of stability or mislocalization of the mutant ODR-7 proteins. Missense mutations in additional domains of ODR-7 did not affect its autoregulatory properties. Although NRs have been shown to regulate target genes in the absence of direct DNA contact (PORTER *et al.* 1997; SCHULE *et al.* 1990; YANG-YEN *et al.* 1990), the requirement for P-box residues suggests that DNA binding by ODR-7 may be necessary for autoregulation.

Residues in the DBD differentially affect the regulation of genes required for the responses to odorants and autoregulation: ODR-7 promotes the expression of AWA-specific signaling genes including the *odr-10* diacetyl receptor and the *osm-9* TRPV-like channel genes (SENGUPTA *et al.* 1996) (P. SENGUPTA, unpublished observations). In the absence of expression of these genes, animals fail to respond to the volatile odorants diacetyl and pyrazine. Mutants that failed to autoregulate also failed to respond to AWA-sensed odorants (Figure 3), suggesting that maintenance of ODR-7 expression through adult stages may be required for the rescue of the diacetyl and pyrazine chemotaxis phenotypes. In principle, ODR-7 could activate the expression of both its own promoter and those of downstream signaling genes via similar mechanisms. However, *odr-7(ky55)* mutants specifically fail to respond to diacetyl while retaining additional wild-type ODR-7 functions, including maintenance of *odr-7* expression (SENGUPTA *et al.* 1994; SAGASTI *et al.* 1999; P. SENGUPTA, unpublished observations), suggesting that distinct residues of ODR-7 may be required for the regulation of different target genes.

In addition to the G340 residue that is mutated in *odr-7 (ky55)*, we identified a second residue that appears to be required specifically for the regulation of genes essential for diacetyl chemotaxis but not for other ODR-7-mediated functions. Residues in the D box in the second zinc finger have been implicated in dimerization (UMESONO and EVANS 1989; DAHLMAN-WRIGHT *et al.* 1991; LUISI *et al.* 1991; RASTINEJAD *et al.* 1995). Transgenic animals from multiple lines expressing an ODR-7(R372A) mutation in the putative D box (Figure 1A) failed to respond to diacetyl while responding normally to pyrazine (Figure 3). Moreover, staining with anti-ODR-7 antibodies showed that ODR-7(R372A) was localized to the nuclei and that levels of ODR-7(R372A) were less than twofold different from those of wild-type ODR-7 (Figure 2 and data not shown). This indicates that the differential regulation of target genes is likely not due to a requirement for different thresholds of ODR-7.

NRs have been shown to contain a bi- or tripartite nuclear localization sequence (NLS) consisting of two or three clusters of basic residues C-terminal to the second zinc finger of the DBD (PICARD and YAMAMOTO 1987; GUIOCHON-MANTEL *et al.* 1989). Mutating the basic residues (K393A/R394G) in the cluster comprising

a putative NLS immediately C-terminal to the second zinc finger of ODR-7 (Figure 1A) completely abolished the ability of ODR-7 to rescue both the diacetyl and pyrazine chemotaxis defects, although autoregulation was unaffected (Figures 2 and 3). ODR-7(K393A/R394G) was localized to the nucleus at levels comparable to those in wild-type animals (Figure 2), indicating that nuclear localization of ODR-7 is mediated by additional residues or via alternate mechanisms in the AWA neurons. These results suggest that ODR-7 uses different mechanisms for the activation of expression of its own promoter and for the expression of downstream signaling genes.

The molecular requirements for repression of *str-2* expression are distinct from those required for activation of gene expression in the AWA neurons: In addition to activating gene expression, ODR-7 also represses expression of the AWC-specific olfactory receptor gene *str-2* in the AWA neurons (Sagasti *et al.* 1999). To determine whether the requirements for activation and repression of gene expression are distinct, we examined the ability of mutant ODR-7 proteins to repress the ectopic expression of *str-2* in the AWA neurons in *odr-7 (ky4)* mutants.

Surprisingly, both ODR-7ΔDBD and ODR-7ΔNTD1 repressed *str-2* expression, suggesting that either domain may be sufficient for this function (Figure 2). The ability of these mutant proteins to repress *str-2* expression was particularly unexpected since neither protein is able to maintain *odr-7* expression. This implies that in contrast to the requirement for ODR-7 throughout development for the regulation of genes necessary for diacetyl and pyrazine chemotaxis, expression of ODR-7 early in development may be sufficient for repression of *str-2* expression in the AWA neurons. As expected, ODR-7ΔNTD2, ODR-7ΔNTD3, and ODR-7ΔCoR also significantly repressed *str-2* expression (Figure 2).

We further dissected the molecular requirements for repression by examining the ability of missense mutations in the ODR-7 DBD to repress ectopic expression of *str-2* in the AWA neurons. Of the mutants examined, only the R356E mutation in the conserved FFRR quartet completely abolished the ability of ODR-7 to repress *str-2* (Figure 2). Both A349E and A350V mutations in the P box that abolished autoregulation retained the ability to repress *str-2* expression, consistent with the hypothesis that ODR-7 acts early in development to regulate *str-2* expression. These results also indicate that the molecular requirements for repression and activation of gene expression are distinct in the AWA neurons.

The molecular requirements for regulation of *str-2* expression are distinct in the AWA and AWC olfactory neurons: Since ODR-7 promotes the expression of AWA-specific genes and represses *str-2* expression in the AWA neurons, we determined whether misexpression of *odr-7* in the AWC neurons was sufficient to repress *str-2* expression and to drive ectopic expression of AWA-specific genes. An *odr-1* promoter drives expression of a green fluorescent protein (*gfp*) reporter gene strongly in the AWC and weakly in the AWB olfactory neurons (L'Etoile and Bargmann 2000). Expression of an *odr-7* cDNA under the *odr-1* promoter resulted in strong repression of *str-2* expression in the AWC neurons (2 AWC^{OFF}; Figure 2) However, AWA-specific genes such as *odr-10* and *odr-7* were not ectopically expressed (data not shown).

We next determined whether ODR-7 repressed *str-2* expression via similar mechanisms in the AWA and AWC neurons. An R356E mutation in the conserved FFRR quartet completely abolished the ability of ODR-7 to repress *str-2* in the AWC neurons, similar to its function in the AWA neurons (Figure 2). ODR-7(R356E) was localized to the nucleus and expressed at levels similar to those of transgenic animals misexpressing wild-type ODR-7 in the AWC neurons (Figure 2). In contrast to the observed phenotypes in the AWA neurons, we found that an E403Q mutation in the predicted T box and the K393A/R394G mutation in the putative NLS failed to significantly repress *str-2* expression in the AWC neurons (Figure 2). T-box residues have been implicated in determining binding site specificity and dimerization (Dahlman-Wright *et al.* 1991; Luisi *et al.* 1991; Wilson *et al.* 1992; Lee *et al.* 1993; Rastinejad *et al.* 1995). Although the levels and the subcellular localization of the E403Q mutant in the AWC neurons were similar to those of animals expressing the wild-type *odr-7* cDNA under the *odr-1* promoter, we found that the ODR-7 (K393A/R394G) protein was present in both the cytoplasm and the nuclei of the AWC neurons as revealed by staining with anti-ODR-7 antibodies (Figure 2). To determine whether nuclear localization of this mutant protein was sufficient to restore *str-2* repression, we expressed ODR-7(K393A/R394G) fused to an NLS from the SV40 large T antigen (Kalderon *et al.* 1984). This fusion protein was localized to the nucleus and weakly repressed *str-2* expression (Figure 2). This result indicates that nuclear localization of ODR-7 is mediated primarily by the K393/R394 residues in the AWC neurons. In addition, the mutated residues may play a role in the repression of *str-2* expression in the AWC but not the AWA neurons.

Consistent with the K393/R394 residues in the DBD being required for nuclear localization of ODR-7 in the AWC neurons, deletion of the DBD resulted in mislocalization of the ODR-7 protein to the cytoplasm and failure to repress *str-2* expression (Figure 2). Forced localization of the ODR-7ΔDBD protein to the nucleus via the addition of the SV40 NLS also failed to repress *str-2* expression, indicating that in contrast to the ability of either the DBD or the NTD to repress *str-2* expression in the AWA neurons, the DBD is essential for *str-2* repression in the AWC neurons. However, expression of the DBD alone (ODR-7ΔNTD1) was not sufficient to repress *str-2* expression. Instead, unexpectedly, expression of ODR-7ΔNTD1 resulted in the expression of *str-2* in both

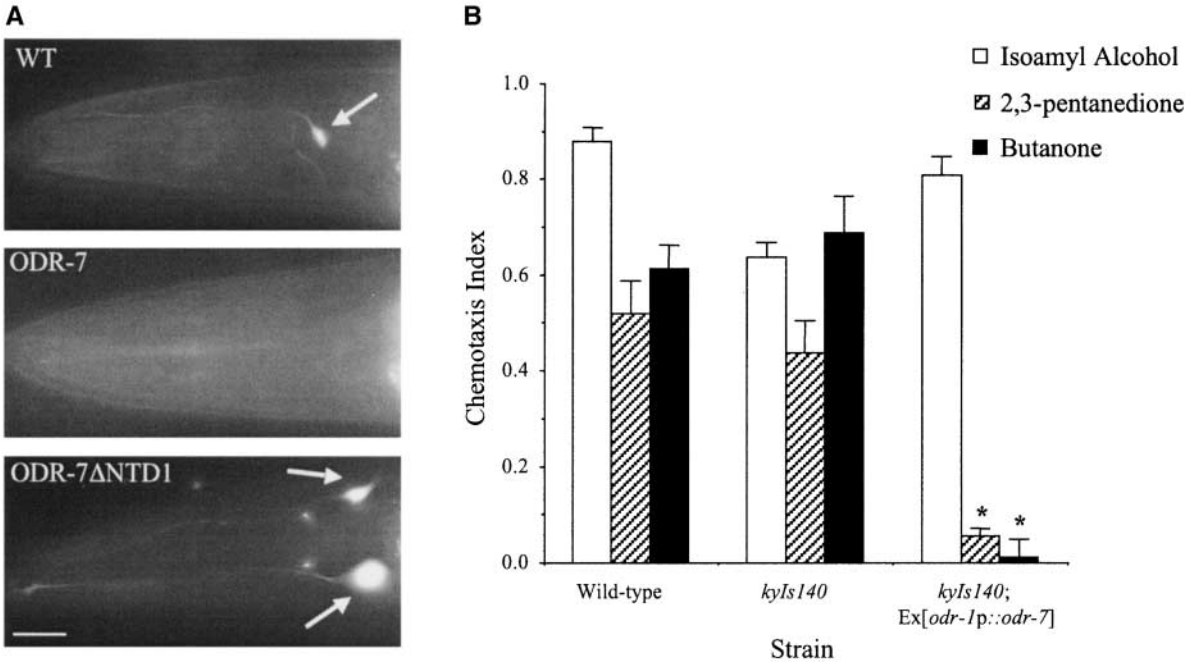


FIGURE 4.—Misexpression of ODR-7ΔNTD1 affects AWC neuronal asymmetry. (A) *str-2*p*::gfp* is expressed in a single AWC neuron (arrow) in wild-type animals. *str-2*p*::gfp* expression is repressed in transgenic animals expressing ODR-7 (middle) and is expressed in both AWC neurons in transgenic animals expressing ODR-7ΔNTD1 (bottom). Anterior is at left; bar, 20 μm. (B) Responses of the indicated strains to a panel of AWC-sensed odorants. Multiple copies of a *str-2*p*::gfp* transgene are stably integrated in the *kyIs140* strain. The concentrations of odorants at the peaks of the gradients were 1 μl of a 1:100 dilution of isoamyl alcohol, 1 μl of a 1:10,000 dilution of 2,3-pentanedione, and 1 μl of a 1:1000 dilution of butanone. The data represent the mean of the responses of two independent assays using ~80–100 animals in each assay. For *kyIs140;* Ex[o*dr-1*p*::odr-7*], transgenic animals from two independent lines were assayed. Responses different from those of *kyIs140* animals at $P < 0.005$ are indicated by an asterisk.

AWC neurons (2 AWC^{ON}; Figures 2 and 4A). ODR-7 ΔNTD1 was localized to the nucleus, similar to the wild-type ODR-7 protein (Figure 2). We were unable to examine the effects of ODR-7ΔNTD2 on *str-2* expression since ODR-7ΔNTD2 appeared to be unstable in the AWC neurons. However, neither ODR-7ΔNTD3 nor ODR-7ΔCoR affected the ability of ODR-7 to repress *str-2* expression (Figure 2). These results show that ODR-7 represses *str-2* expression via distinct molecular mechanisms in the AWA and AWC neurons.

ODR-7-mediated regulation of *str-2* expression in the AWC neurons requires cGMP but not mitogen-activated protein kinase signaling: The left and right AWC neurons mediate sensory responses to chemicals such as isoamyl alcohol and both neurons express a defined subset of signaling genes (BARGMANN *et al.* 1993; TROEMEL 1999; WES and BARGMANN 2001). In addition to these bilaterally symmetric functions, the left and right AWC neurons each mediate distinct sensory responses. *str-2* acts as a marker for these asymmetric fates. Thus, the AWC neuron expressing *str-2* (AWC^{ON} neuron) is required for attraction to the odorant butanone, while the AWC^{OFF} neuron is required for chemotaxis toward the odorant 2,3-pentanedione (WES and BARGMANN 2001). Calcium signaling via the UNC-43 CaMKII, the NSY-1 mitogen-activated protein kinase kinase kinase (MAPKKK), and the SEK-1 MAPKK initially represses *str-2* expression in both AWC neurons likely via modulation of activity of a transcriptional repressor (TROEMEL *et al.* 1999; SAGASTI *et al.* 2001; TANAKA-HINO *et al.* 2002). An unidentified lateral signal requiring axo-axonal contact between the two bilateral AWC neurons inhibits calcium signaling, resulting in *str-2* expression in one of the two AWC neurons in a stochastic manner. Subsequently, *str-2* expression is maintained in the AWC^{ON} neuron via cGMP signaling (TROEMEL *et al.* 1999). We examined where heterologously expressed ODR-7 acts in this pathway to regulate *str-2* expression in the AWC neurons.

str-2 is expressed in both AWC neurons in *nsy-1* mutants (2 AWC^{ON} phenotype; TROEMEL *et al.* 1999; SAGASTI *et al.* 2001). Expression of an *odr-1*p*::odr-7* transgene resulted in a 2 AWC^{OFF} phentoype in *nsy-1* mutants (Table 1), suggesting that ODR-7 does not require NSY-1 function to repress *str-2* expression. Loss-of-function mutations in the guanylyl cyclase gene *odr-1* result in a failure to maintain *str-2* expression (2 AWC^{OFF} phenotype; TROEMEL *et al.* 1999). Since expression of ODR-7 ΔNTD1 results in a 2 AWC^{ON} phenotype, we determined whether ODR-7ΔNTD1 expression is epistatic to *odr-1 (lof)*. We found that although *odr-1* mutants transgenic for ODR-7ΔNTD1 expressed *str-2* in both AWC neurons

TABLE 1

ODR-7-mediated regulation of *str-2* expression in the AWC neurons

	% expressing *str-2*p*::gfp* in no. of AWC neurons		
Strain	None	One	Two
Wild type	0	100	0
Ex[*odr-1*p*::odr-7*]	82.2	17.8	0
Ex[*odr-1*p*::odr-7ΔNTD1*]	1.9	45.8	52.3
nsy-1(ky397)	0	0	100
nsy-1(ky397); Ex[*odr-1*p*::odr-7*]	66.1	29.9	4
odr-1(n1936)	100	0	0
odr-1(n1936); Ex[*odr-1*p*::odr-7ΔNTD1*]	96.0	4.0	0

All strains contain integrated arrays of a *str-2*p*::gfp* transgene. For transgenic lines, data shown are from at least two independent lines. $n > 90$ for each. Adult animals were examined.

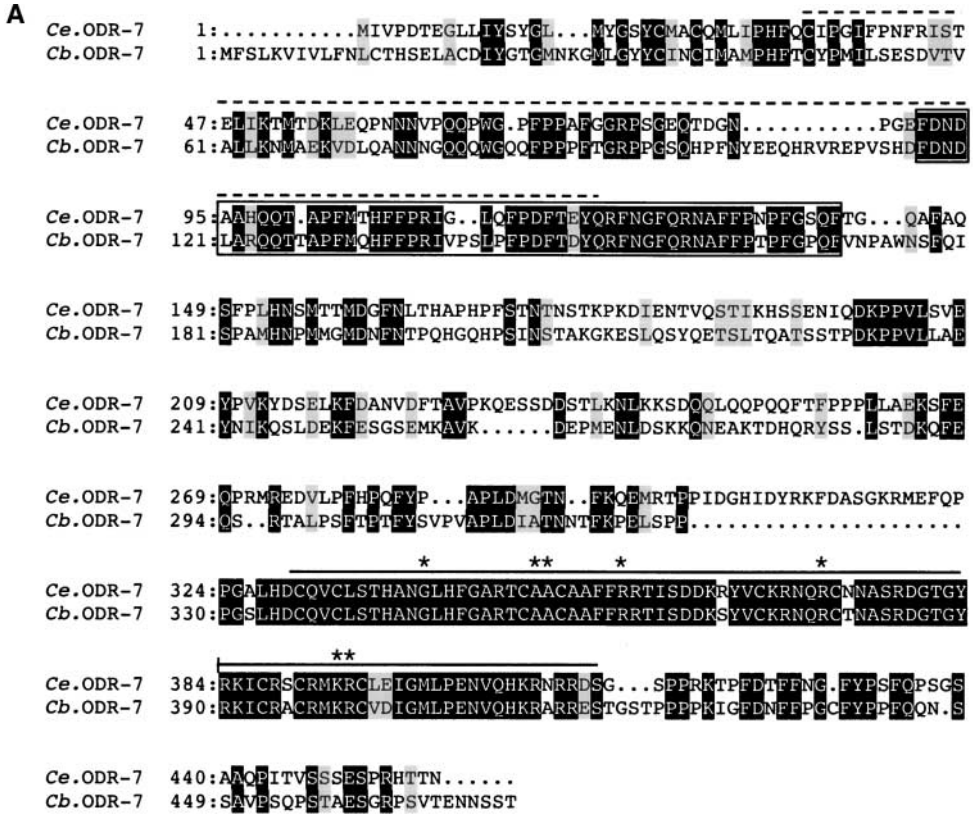
in early larval stages, expression was not maintained in adults (Table 1). Similarly, maintenance of ectopic *str-2* expression in the AWA neurons in *odr-7* mutants also required *odr-1* (M. E. Colosimo and P. Sengupta, unpublished results). This result suggests that cGMP signaling is required for the maintenance of ODR-7-regulated *str-2* expression in both the AWA and AWC neurons.

ODR-7-mediated regulation of *str-2* expression in the AWC neurons could result from defects in guidance of the AWC neurons and failure to initiate or maintain axo-axonal contact. Although we cannot completely rule out this possibility, we found that transgenic animals expressing either full-length ODR-7 or ODR-7ΔNTD1 retained normal responses to AWC-sensed odorants such as isoamyl alcohol (Figure 4B and data not shown), suggesting that overall AWC cell fate, synaptic connectivity, and morphology are not grossly altered upon overexpression of these transgenes. To determine whether misexpression of ODR-7 also affects the asymmetric sensory functions of the AWC^{ON} and AWC^{OFF} neurons, we examined the chemosensory responses of transgenic animals expressing the *odr-1*p*::odr-7* fusion gene. We found that *odr-1*p*::odr-7*-expressing transgenic animals exhibited strong defects in their response to butanone, consistent with their 2 AWC^{OFF} phenotype (Figure 4B). However, these animals also exhibited defects in their responses to 2,3-pentanedione. This suggests that in addition to regulating *str-2* expression, misexpression of ODR-7 also results in alterations in specific sensory functions of the AWC neurons.

The *C. briggsae odr-7* gene can substitute for *odr-7* in *C. elegans* and is expressed in the AWA neurons: Examination of the recently released *C. briggsae* genomic sequence revealed a putative ortholog of *odr-7*. Similar to the *C. elegans* ODR-7, the DBD of the *C. briggsae* ortholog is located near the C terminus of the protein (Sengupta *et al.* 1994). An alignment of the ODR-7 protein sequences of the two species showed that the DBD was highly conserved, with 91% identity between the predicted proteins (Figure 5A). Moreover, all residues shown to be required for the functions of ODR-7 in the AWA neurons are conserved in *C. briggsae* (Figure 5A). The NTDs were more divergent with only 42% identity. However, within the NTD, a 51-aa domain showed a high degree of conservation (84% identity; Figure 5A). Interestingly, residues within this highly conserved domain were identified as being required for maintenance of *odr-7* expression in *C. elegans*. These results suggest that the molecular mechanisms of ODR-7 function may be conserved between the *C. elegans* and *C. briggsae* ODR-7 proteins.

To determine whether the functions of ODR-7 were conserved, we examined the olfactory responses of *odr-7 (ky4)* mutants expressing *C. briggsae odr-7* genomic sequences. Transgenic animals responded normally to both diacetyl and pyrazine (Figure 3), indicating that *C. briggsae odr-7* can substitute for *odr-7* functions in *C. elegans*. Moreover, a fusion gene carrying 4.5 kb of *C. briggsae odr-7* promoter sequences fused to *gfp* drove expression solely in the AWA neurons in *C. elegans*, similar to the expression pattern of the *C. elegans odr-7* gene (Figure 5B). The *C. elegans odr-7*p*::gfp* fusion gene was also expressed in *C. briggsae* in a bilateral pair of neurons whose relative positions corresponded to the positions of the AWA neurons in *C. elegans* (Figure 5B). These results indicate that both the expression pattern and functions of *odr-7* are conserved between *C. elegans* and *C. briggsae*.

NHR-74 can substitute for ODR-7 in repressing *str-2* expression but not in maintenance of *odr-7* expression: To investigate whether other members of the divergent NR family in *C. elegans* are able to substitute for ODR-7 functions, we expressed an *nhr-74* cDNA under the *odr-7* promoter in *odr-7(ky4)* null mutants. NHR-74 contains a P box identical to that of ODR-7 and was previously shown to be expressed in the hypodermal seam cells (Miyabayashi *et al.* 1999). NHR-74 was unable to maintain expression of an *odr-7*p*::gfp* transgene (Figure 2). Since sequences in the NTD of ODR-7 are also required



	% identity	% similarity
Whole	50%	59%
DBD	91%	95%
NTD	42%	53%

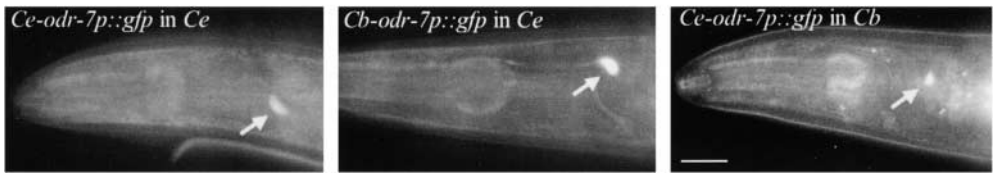


FIGURE 5.—The sequence and expression patterns of the *C. elegans* and *C. briggsae odr-7* genes are conserved. (A) An alignment of the *C. elegans* and *C. briggsae* ODR-7 proteins generated using ClustalW (HIGGINS *et al.* 1996). Residues with a black background are identical; residues with a gray background are similar. The DBD and the residues deleted in ODR-7ΔNTD3 are indicated with solid and dashed overbars, respectively. The boxed region indicates a domain of high homology in the NTDs. Percentages of identity and similarity were calculated using the Blast2 application (TATUSOVA and MADDEN 1999). Asterisks denote conserved residues mutated in this analysis. (B) The expression patterns of the *Ce-odr-7*p::*gfp* or the *Cb-odr-7*p::*gfp* transgenes in the indicated strains. Arrow points to the cell body of an AWA neuron. Anterior is at left; bar, 20 μm.

for autoregulation and NHR-74 does not share sequence homology with ODR-7 in domains other than the DBD, we determined whether a fusion protein between the NTD of ODR-7 and the DBD of NHR-74 could activate expression from the *odr-7* promoter. As shown in Figure 2, this fusion protein also failed to maintain *odr-7*p::*gfp* expression, indicating that residues specific to the ODR-7 DBD are essential for autoregulation.

However, NHR-74 repressed *str-2* expression in the AWA neurons (Figure 2). Moreover, expression of NHR-74 in the AWC neurons under the *odr-1* promoter also resulted in significant repression of *str-2* expression in the AWC neurons (Figure 2). We determined whether expression of the NHR-74 DBD alone would result in a 2 AWC^{ON} phenotype similar to the phenotype observed upon expression of the ODR-7ΔNTD1 protein. As shown in Figure 2, expression of the NHR-74 DBD (NLS::NHR-74DBD) repressed *str-2* expression but did not result in a 2 AWC^{ON} phenotype.

DISCUSSION

We have exploited our knowledge of the multiple functions of ODR-7 to define the residues and domains required for each regulatory role *in vivo*. Our results indicate that ODR-7 utilizes multiple mechanisms to regulate distinct sets of target genes and that these mechanisms are different in different cell types. The results are summarized in Figure 1A.

ODR-7 function in the AWA neurons: In the AWA neurons, ODR-7 promotes its own expression, as well as the expression of genes required for chemotaxis to

the volatile odorants diacetyl and pyrazine. The P boxes of all nonsteroid NRs in vertebrates contain the sequence CXGCKG. Since the P box of ODR-7 has the unusual sequence CAACAA, it was formally possible that ODR-7 mediates its functions in the absence of direct DNA contact (Nelson *et al.* 1993; Bjornstrom and Sjoberg 2002). We have now shown that P-box residues, as well as the FFRR basic sequence motif following the first zinc finger, are critical for the autoregulatory functions of ODR-7, suggesting that ODR-7 directly binds a response element in its own promoter to maintain expression. However, the P box and the basic quartet are not sufficient for autoregulation, since the NHR-74 DBD that contains an identical P box and FFRR quartet is unable to substitute for the ODR-7 DBD in autoregulation. Interestingly, a domain in the NTD is also critical for autoregulation. Residues in the C-terminal LBDs of NRs such as the 9-*cis* retinoic acid receptor RXR and HNF4 play major roles in both hetero- and homodimerization of NRs in solution (Marks *et al.* 1992; Bourguet *et al.* 1995; Bogan *et al.* 2000). NTD residues deleted in ODR-7ΔNTD3 may play similar roles in enabling ODR-7 to bind its promoter as either a homo- or heterodimer with an as yet unidentified factor. These required NTD residues are highly conserved in the *C. briggsae* ortholog, suggesting that ODR-7 may utilize similar mechanisms to maintain expression in *C. briggsae*.

Although the requirement for autoregulation precluded our ability to examine the effects of several mutations on the regulation of genes required for diacetyl and pyrazine chemotaxis, we identified a subset of residues required specifically for regulation of diacetyl chemotaxis. Mutation of a well-conserved Gly (G340) in the first zinc finger and an Arg (R372) in the D box in the second zinc finger specifically abolished the ability of ODR-7 to regulate genes required for chemotaxis to diacetyl. A role for the conserved G340 residue has not previously been reported in the described structures of the NR DBDs bound to DNA. However, this residue is adjacent to residues shown to contact the phosphate backbone of DNA, suggesting that it may play a role in DNA binding by ODR-7. Since residues in the D box have been implicated in both homo- and heterodimerization of NRs (Umesono and Evans 1989; Hard *et al.* 1990; Luisi *et al.* 1991; Schwabe *et al.* 1993; Zechel *et al.* 1994; Rastinejad *et al.* 1995), ODR-7 may regulate genes required for diacetyl chemotaxis by heterodimerizing with a partner. In addition, basic residues C-terminal to the zinc finger are required for the regulation of genes required for both diacetyl and pyrazine chemotaxis. Since mutations in this basic cluster do not affect autoregulation, this indicates that ODR-7 utilizes at least a subset of distinct mechanisms for autoregulation and for the regulation of additional target genes. Taken together, this mutational analysis highlights an unexpected diversity of mechanisms by which ODR-7 mediates its multiple roles in the AWA neurons.

Early and late requirements for ODR-7 function in the AWA neurons: Our results also enabled the dissection of early and late roles of ODR-7 in the functional specification of the AWA neurons. *odr-7* expression in the AWA neurons is initiated by the LIM homeodomain protein LIN-11 whose expression is downregulated by early L1 stages (Sarafi-Reinach *et al.* 2001). *odr-7* expression is subsequently maintained by autoregulation. All mutations that abolished the autoregulatory functions of ODR-7 also failed to rescue the diacetyl and pyrazine chemotaxis defects, suggesting that maintenance of *odr-7* expression through adult stages may be necessary for the regulation of expression of genes required for these behaviors. However, mutations such as A349E and A350V that abolished autoregulation could still repress *str-2* expression in the AWA neurons. This observation indicates that expression of *odr-7* prior to the L1 stage is sufficient for repression of *str-2* expression. In both the AWB and AWC olfactory neurons, the expression pattern of *str-2* is also specified during early embryonic stages (Sagasti *et al.* 1999; Troemel *et al.* 1999). Thus, ODR-7 acts to regulate distinct sets of target genes both during early and late development of the AWA neurons.

ODR-7-mediated regulation of *str-2* expression in the AWA and AWC neurons: The molecular requirements for ODR-7-mediated repression of *str-2* expression appear to be distinct from the requirements for activation of expression of *odr-7* and genes required for odorant responses. Moreover, these requirements appear, at least in part, to be different between the AWA and AWC neurons. However, the mechanism by which ODR-7 represses *str-2* expression is unclear. ODR-7 may act directly as a repressor or activate the expression of a repressor. Alternatively, ODR-7 may interfere with the function of an activator required for *str-2* expression. *str-2* expression has also been shown to be regulated by axo-axonal contact and calcium signaling in the AWC neurons (Troemel *et al.* 1999; Sagasti *et al.* 2001; Tanaka-Hino *et al.* 2002). It is possible that ODR-7 represses *str-2* expression in the AWA neurons by altering similar signaling pathways.

Regardless of the mechanism, the molecular requirements for *str-2* repression are clearly distinct from those required for the activation of other target genes. P-box residues are not required for *str-2* repression in either the AWA or AWC neurons, suggesting that direct contact with specific bases in a cognate response element is not essential for repression. However, R356 is essential for repression in both cell types. Since this residue has been implicated in both direct and indirect contact with DNA, a simple hypothesis suggests that ODR-7 interacts with other transcription factors to regulate *str-2* repression and that this interaction does not require P-box-mediated binding site recognition. NRs have been shown to regulate target genes in the absence of DNA binding via interaction with other transcription factors binding to their cognate sites (Porter *et al.* 1997; Schule

et al. 1990; Yang-Yen *et al.* 1990). Similarly, binding specificity may be provided by an interacting partner of ODR-7, although the FFRR sequence of ODR-7 is likely important either for correct localization of the complex on DNA or for stabilization of the complex. However, in the AWA neurons, this hypothesis is complicated by the observation that both the NTD and the DBD are sufficient to repress *str*-2 expression. We speculate that ODR-7 interacts with its partner via either its NTD or DBD in the AWA neurons and that the R356E mutation results in a change in the stability or conformation of the protein, preventing this interaction. Moreover, since a T-box residue (E403) is required for *str-2* repression in the AWC but not in the AWA neurons, ODR-7 may interact with partner protein(s) via a mechanism requiring the T box in the AWC neurons.

An unexpected observation was that while expression of ODR-7 resulted in repression of *str-2* expression in both AWC neurons, expression of only the ODR-7 DBD resulted in a 2 AWC^{ON} phenotype. Calcium and MAP kinase signaling in an AWC neuron are essential for *str-2* repression likely via modulation of activity of a transcription factor (Troemel *et al.* 1999; Sagasti *et al.* 2001; Tanaka-Hino *et al.* 2002). However, ODR-7 is able to repress *str-2* expression in the absence of MAP kinase signaling since ODR-7-mediated repression of *str-2* expression is unaffected in *nsy-1* mutant animals. The ODR-7 DBD may bind to and/or compete away either the repressing factor itself or proteins required for the repression function, resulting in a 2 AWC^{ON} phenotype. Expression of the NHR-74 DBD did not result in a 2 AWC^{ON} phenotype, indicating that residues other than those conserved between the NHR-74 and ODR-7 DBD are important for this process. These results raise the intriguing possibility that repression of *str-2* expression in an AWC neuron may be mediated by an NR. MAP kinase signaling has been shown to modulate the functions of several NRs (Kato *et al.* 1995; Hu *et al.* 1996; Lange *et al.* 2000). In the *str-2*^{OFF} neuron, calcium and MAP kinase signaling may similarly phosphorylate an NR to result in *str-2* repression. It will be interesting to determine if this is indeed the case and whether this NR is expressed asymmetrically or activated asymmetrically in an AWC neuron.

Functions of additional divergent NRs in *C. elegans*: Is ODR-7 representative of the large divergent class of NRs in *C. elegans*? Despite the identities in the P-box residues of ODR-7 and NHR-74, full-length NHR-74 and an ODR-7NTD::NHR-74DBD fusion protein are unable to maintain expression of an *odr-7*p*::gfp* transgene, suggesting that additional nonconserved residues are required for this function. Although both NHR-74 and ODR-7 are able to repress *str-2* expression, it is unclear whether these proteins mediate this function via similar molecular mechanisms. Since ODR-7 is evolutionarily unique, we suggest that ODR-7 utilizes relatively novel mechanims to regulate gene expression. However, it remains possible that ODR-7 may share functions with additional nematode-specific divergent NRs.

It has been suggested that the multitude of NRs encoded by the *C. elegans* genome responds to specific environmental signals or internal metabolites, so as to coordinate and fine tune changes in behavior or development (Yamamoto 1997; Sluder *et al.* 1999). Although the functions of a subset of these divergent NRs may be regulated by ligands, the NTD of ODR-7 does not share either sequence or structural homology to the LBDs of other NRs that are known to be ligand regulated, and the NTD appears to be dispensable for a subset of its functions. Thus, ODR-7 and perhaps a subset of additional divergent NRs in *C. elegans* may act as ligand-independent transcription factors.

Dissection of the functions of ODR-7 *in vivo* has revealed a surprising diversity of mechanisms by which ODR-7 regulates target gene expression. Gene duplication and divergence has been proposed to be a major force driving the evolution of new species (Ohno 1970). Extensive duplication and diversification of NRs may have played an important role in the speciation of nematodes. This analysis is a first step toward the elucidation of divergent NR function *in vivo*. An important goal for the future will be to further investigate these gene-regulatory mechanisms and to determine whether other divergent NRs utilize similar mechanisms to mediate their as yet unknown functions.

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